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REMARKS

Reconsideration of this application is respectfully requested.

Claims 29-31 were rejected under 35 U.S.C. § 101 and under 35 U.S.C. § 112, first paragraph, for allegedly not being supported by a well-established utility. Although the Examiner concedes that the prior art describes using immune complexes to induce immune responses, the Examiner contends that this utility is not disclosed in the instant application. (Office Action at 1-2.) Applicants traverse the rejection.

The lack of a specific statement of utility in the specification does not negate utility *per se*. M.P.E.P. 2107.02(II)(B). Rather, if an invention has a well-established utility, rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, based on lack of utility should not be imposed. *Id.* (citing *In re Folkers*, 344 F.2d 970, 145 U.S.P.Q. 390 (C.C.P.A. 1965)). Applicants' claimed immune complexes have a well-established utility, namely, they can be used to generate immune responses. The Examiner has not asserted that applicants' immune complexes could not be used in this well-established utility. In fact, the Examiner has recognized that this utility for immune complexes can be found in the prior art. (Office Action at 1-2.) There is no reason to believe that applicants' immune complexes could not be used to induce immune responses. Accordingly, the rejection of applicants' claimed immune complexes for lacking utility is improper. *See id.*

Furthermore, applicants provide herewith objective evidence (Exhibits 1-3) that immune complexes were well-known to be useful for the generation of immune responses prior to the

filing date of this application. In Nishi, 1970 (Exhibit 1), immune complexes (antibody-α_f-globulin) were used to immunize a horse. Nishi at 2507, col. 2. The serum from the horse showed a highly-specific reaction for α_f-globulin. *Id.* at 2509, col. 1. In addition, Higgins, 1980, (Exhibit 2), indicates that immune complexes can be used "as immunogens to elicit the formation of a more highly specific antisera." Higgins at 889, col. 1. In this paper, agarose-immobilized immune complexes were used to immunize a rabbit. Higgins at 889, col. 2. The antiserum generated against the immune complexes was higher titer than the original antiserum. *Id.* Moreover, Eager et al., 1983 (Exhibit 3), indicate that immunization with immune complexes can be an effective way to produce monoclonal antibodies. Eager et al. at 157. This paper indicates that immune complexes acted as effective immunogens in both mice and rats, and the advantages of using this method for immunization are discussed. *Id.* at 163. The skilled artisan would immediately appreciate that applicants' immune complexes were likewise useful. Thus, applicants' claimed invention has a well-established utility. Accordingly, applicants respectfully request withdrawal of the rejections.

Claims 29-31, 39, 40, and 45-49 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject mater that was not described in the specification in such a way as to reasonably convey to the skilled artisan that the inventors had possession of the claimed invention at the time the application was filed. The Examiner does not dispute that the Declaration of Dr. Cohen under 37 C.F.R. § 1.132 demonstrates that the skilled artisan would be capable of following the teachings of the disclosure to prepare the claimed invention. However,

the Declaration is deemed insufficient by the Examiner because "[t]there is nothing in the disclosure to suggest to the skilled artisan that applicants actually prepared the claimed immune complexes or antibodies." (Office Action at 4-5.) The Examiner indicates that applicants may obviate the rejection by providing scientific evidence that the claimed antibodies and immune complexes were actually generated. (Office Action at 4.) Applicants traverse the rejection.

The Examiner's insistence on the actual preparation of the claimed immune complexes or antibodies (i.e., a working example) to fulfill the written description requirement of 35 U.S.C. § 112, first paragraph, is contrary to current legal precedent. As explained in applicants' January 21, 2003, Amendment and Response to Paper No. 51, decisions by both the Court of Appeals for the Federal Circuit (*Gould v. Quigg*) and the Supreme Court (*Pfaff v. Wells Electronics, Inc.*) dictate that a working example is ***not required*** for applicants to fulfill the written description requirement of 35 U.S.C. § 112, first paragraph. Applicants respectfully request that the Examiner explain how his requirement for a working example can be maintained in view of these two cases.

Moreover, the Examiner's position is untenable in view of the recent decision in *Noelle v. Lederman*, 355 F.3d 1343, 1349, 69 U.S.P.Q.2d 1508, 1514 (Fed. Cir. 2004). In *Noelle*, the Court of Appeals for the Federal Circuit found that an applicant can claim an antibody by its binding affinity to an antigen if the applicant has disclosed a fully characterized antigen either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository. *Id.* Thus, in *Noelle*, it was not necessary for the applicant to have "actually

prepared" the antibody to claim it. It was sufficient to have fully described the antigen, and such a description could be accomplished through a deposit of the antigen. The same is true of applicants' claimed antibodies and immune complexes. The Examiner's requirement for applicants to have "actually prepared" the antibodies and immune complexes to claim them (i.e., a working example) runs counter to the decision in *Noelle*, and is in error.

According to *Noelle*, applicants do not need to have a working example of making antibodies against the claimed antigens to meet the written description requirement. Rather, they can meet the requirements of 35 U.S.C. § 112, first paragraph, by disclosing a "fully characterized antigen" either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository. *See id.* Applicants' specification fulfills this requirement. Applicants deposited isolates of HIV-1 at the Collection Nationale des Cultures de Micro-organismes (specification at 27), and described how to propagate HIV-1 and isolate the antigens recited in claims 29-31, 39, 40, and 45-49 (specification at 10-13). Since the deposited virus contains the recited antigens, applicants' deposit should be viewed as fulfilling the written description requirement of 35 U.S.C. § 112, first paragraph, for antibodies against these "fully characterized antigens." *See Noelle*, 355 F.3d at 1349, 69 U.S.P.Q.2d at 1514. Accordingly, applicants respectfully request withdrawal of the rejection.

Applicants respectfully submit that this application is in condition for allowance. In the event that the Examiner disagrees, he is invited to call the undersigned to discuss any outstanding issues remaining in this application in order to expedite prosecution.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

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Dated: May 18, 2004

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Isolation and Characterization of a Human Fetal α-Globulin from the Sera of Fetuses and a Hepatoma Patient

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SUMMARY

It has been demonstrated that fetal serum contains an α-globulin which is not present in normal adults. This fetal α-globulin has become the subject of great interest because it appears again specifically in the sera of hepatoma patients. It was isolated from the serum of a patient with hepatoma as well as from fetuses by an immunochemical method. Physicochemical and chemical properties of the two preparations were studied, and essentially identical results were obtained.

INTRODUCTION

Fetal serum of many mammalian species, including humans, contains an α-globulin which is not present in the serum of the normal adult (4, 7, 12, 22, 32). In humans, this fetal-specific protein is found in the serum during the gestational period and is variously called α_f-globulin, postalbumin, α-fetoprotein, α-fetoglobulin, Substance X, etc. (1, 3, 4, 7, 11).

In 1963, Abelev *et al.* (2) first reported that transplantable mouse hepatomas synthesized and secreted into the blood an α-globulin which was immunologically identical to α_f-globulin. Similar observations were made on rats (13) and monkeys (15) with chemically induced primary hepatomas.

Tatarinov and several other investigators (1, 3, 18, 23, 29–31) have shown that α_f-globulin is present in 50 to 80% of patients with hepatomas.

Previous investigations of the physicochemical and chemical natures of these fetal α-globulins have dealt almost exclusively with the calf (22, 25, 26) and rat (17, 27), but to the present human α_f-globulin has not been extensively studied.

The present paper deals with the isolation and characterization of human α_f-globulin from human fetuses and a hepatoma patient.

MATERIALS AND METHODS

Fetal Serum

Human fetuses, ranging from 5 to 7 months gestational period, were obtained by abortion, and blood was taken by heart puncture. Sera of 10 fetuses were pooled.

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Hepatoma Patient Serum

Serum was obtained from a 15-year-old male patient with histologically confirmed hepatocellular carcinoma.

Production of Antisera

Absorption. Rabbits were given s.c. injections of 0.1 ml of the pooled fetal serum emulsified in an equal volume of Freund's complete adjuvant at weekly intervals for 3 injections. The rabbits were bled 2 weeks after the last injection, and the antisera against fetal serum were collected and pooled. The antiserum was absorbed with pooled normal adult serum from 10 persons adding 3 ml of the pooled serum to 1 ml of the antiserum. The absorbed antiserum thus obtained was demonstrated to be specific for α_f-globulin.

Immunization with Antigen-Antibody Precipitate. The absorbed rabbit antiserum specific for α_f-globulin was mixed with the hepatoma patient serum at optimal precipitation proportion. The solution was allowed to react at 37° for 60 min, and formed antigen-antibody precipitate was collected by centrifuging at 3,000 rpm for 15 min and washed with 0.15 M NaCl solution 3 times in the cold. The precipitate was then dissolved in 5 volumes of 0.10 M glycine-HCl buffer, pH 1.8, and the solution was centrifuged at 10,000 rpm for 15 min to remove insoluble material. The pH of the solution was then neutralized to 7.0 with 0.4 M Na_2HPO_4 whereupon the antigen-antibody precipitate reformed. This procedure, i.e., dissolving the antigen-antibody precipitate in acid and precipitating the antigen and the antibody by neutralization, was repeated twice as shown in Chart 1. By this treatment, impurities other than antigen and antibody appeared to be completely removed.

A horse was given an injection s.c. in the side of the neck with the washed precipitate emulsified in Freund's complete adjuvant at intervals of 10 days for 3 injections. For each injection, the precipitate prepared from 1 ml of the hepatoma patient serum (about 0.5 mg of the antigen) was used. Two weeks after the last injection, the horse was bled from the carotid and the antiserum was obtained.

Purification of α_f-Globulin from Antigen-Antibody Precipitate

α_f-Globulin was purified both from the hepatoma patient serum and the pooled fetal serum. Antigen-antibody precipi-

Chart 1

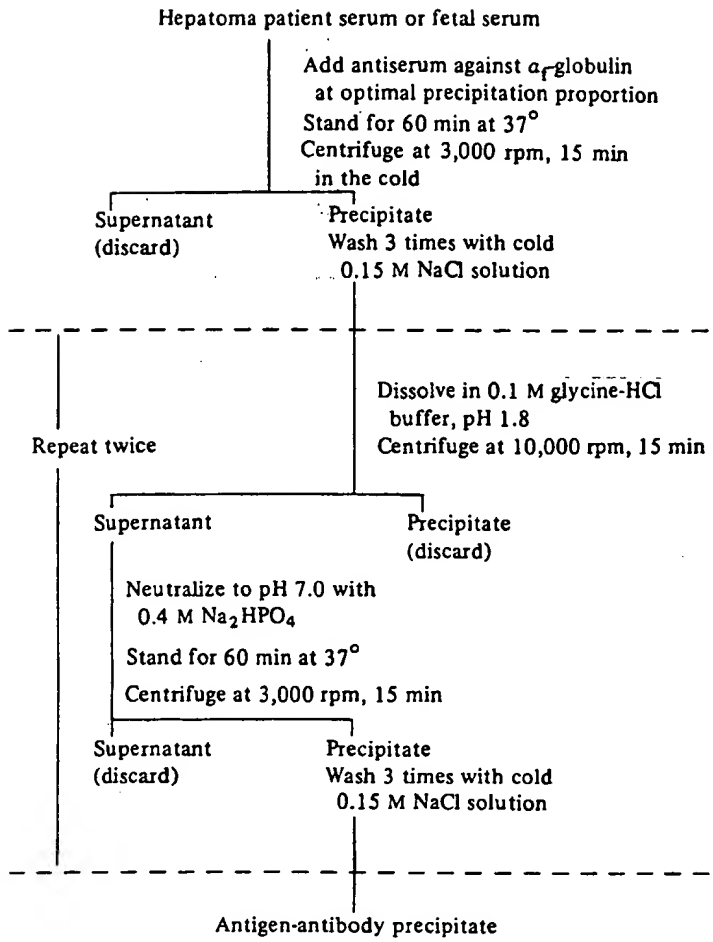

Chart 1. Preparation of antigen-antibody precipitate.

tate was prepared by mixing the horse antiserum and either the fetal serum or the hepatoma patient serum according to the procedure shown in Chart 1.

The precipitate was dissolved in an equal volume of 0.10 M glycine-HCl buffer, pH 1.8, and subjected to gel filtration on Sephadex G-150 column (2.6 x 95 cm) equilibrated with the same buffer. A 5-ml volume of the solution, about 150 mg of antigen-antibody precipitate, was applied to one column. The dissociated antigen and antibody were eluted at a flow rate of 20 ml/hr. The spectrophotometric absorption of the eluate at 280 mμ was constantly monitored, and the eluate was collected in 5-ml fractions. Each fraction was neutralized with 0.4 M Na_2HPO_4 and the presence of α_f-globulin was determined by radial immunodiffusion test as described below. α_f-Globulin containing fractions were pooled and concentrated to 3 ml by ultrafiltration and subjected again to gel filtration to remove a small amount of contaminating antibody. In the 2nd gel filtration, the acidic buffer used in the 1st gel filtration was replaced with phosphate-buffered 0.15 M NaCl solution, pH 7.0.

The purified α_f-globulin thus obtained was dialyzed exhaustively against redistilled water and lyophilized.

Double Immunodiffusion, Immunoelectrophoresis, and Radia Immunodiffusion

The double diffusion method was that described by Ouchterlony (21). The immunoelectrophoretic method wa that described by Grabar and Williams (14). The radia immunodiffusion method was that described by Mancini *et al.* (19). A 1.0% agarose gel in 0.05 M barbital buffer, pH 8.6, was used in the above methods.

Disc Electrophoresis

The disc electrophoretic method was that described by Davis (9).

Physicochemical Analyses

Sedimentation Coefficient. α_f-Globulin dissolved in 0.15 M NaCl solution was examined with a Spinco Model E ultra centrifuge at 56,100 rpm. The sedimentation coefficient wa measured at various concentrations (1.4 to 0.4%) and extrap olated to zero concentration.

Diffusion Coefficient. The diffusion coefficient of a 0.69 α_f-globulin solution was measured in a synthetic boundary cell at 15,220 rpm for 55 min by the method of Schachman as modified by Kawahara (16). Molecular weight was calcu lated from the equation of Svedberg (28). A partial specifi volume calculated from amino acid composition (20) wa used in the above calculations.

Extinction Coefficient. The ultraviolet absorption of α_f globulin was measured in redistilled water with a Hitach Perkin-Elmer 139 UV-VIS spectrophotometer. The concen tration was determined by drying aliquots to constant weigh at 100° over P_2O_5 in a vacuum.

Chemical Analyses

Nitrogen and Sulfur. Nitrogen was determined by a micro Dumas method. Sulfur was determined by the method of Schöniger (24).

Nonnitrogenous Sugars. Nonnitrogenous sugars were determined by the Winzler orcinol-sulfuric acid reaction (10) Glucose was used as standard sugar.

Amino Acid Composition. Amino acid analyses were performed with a Hitachi model KLA-3 automatic amino acid analyzer (6). Hydrolysis was performed in 6 N HCl in an evacuated, sealed tube at 110° for 24 hr. After hydrolysis HCl was removed in a vacuum rotor at 35–40°. Tryptophan and tyrosine were determined from the ultraviolet absorption spectrum of α_f-globulin in 0.1 N NaOH by the method of Bencze and Schmid (5).

RESULTS

Antisera. Rabbit antiserum against fetal serum developed about 10 precipitation lines against either fetal or normal adult sera by immunoelectrophoresis. When absorbed, the antiserum gave only a single precipitation line against the fetal sera in the region between albumin and α_1-globulin bu

did not react with the normal adult sera. An example is shown in Fig. 1. Against hepatoma patient sera, the antiserum gave also a single precipitation line fusing completely with the fetal serum precipitation line. An example is shown in Fig. 2.

The horse antiserum prepared by immunization with the antigen-antibody precipitate also showed the highly specific reaction for α_f-globulin against sera from patients with hepatoma and fetuses. This antiserum also contained antibody against rabbit immunoglobulin, but this did not interfere with the above reaction.

Clinical Data. A few clinical investigations were carried out with the horse antiserum. The prevalence of α_f-globulin was as follows. α_f-Globulin was detected by radial immunodiffusion test in sera of 23 out of 31 patients with primary liver cancer. In 20 histologically confirmed cases, 16 showed positive reactions. Sera of 500 patients with noncancerous liver diseases, hepatitis, cirrhosis, abscess, etc., and sera of 100 patients with malignant tumors of nonliver origin, including liver metastases, gave negative reactions with one exception. This positive serum was from a patient with gastric cancer with massive liver metastasis. Details of the clinical study will be presented elsewhere.

Purification. Antigen-antibody precipitate prepared from either the pooled fetal serum or the hepatoma patient serum was dissolved and subjected to gel filtration. From the 1st gel filtration, 3 peaks were obtained (Chart 2*a*). Peak 1 gave a negative test for antibody and antigen by immunodiffusion test. In addition, this fraction precipitated almost quantitatively when neutralized and appeared to consist of antigen-antibody complex. Peak 2 gave a positive test for antibody and a negative test for antigen and appeared to consist of antibody. Peak 3 gave a negative test for antibody and a positive test for antigen. Radial immunodiffusion test showed that this peak consisted of α_f-globulin.

Peak 3 was again subjected to gel filtration at neutral pH. On the 2nd gel filtration, contaminating antibody formed a soluble antigen-antibody complex because of antigen excess, and 2 peaks were obtained (Chart 2*b*). Both peaks gave a positive test for antigen and a negative test for antibody. The position of Peak 5 in the elution pattern was identical to that of α_f-globulin in the elution pattern of the fetal serum or the hepatoma patient serum. Peak 5 was dialyzed and lyophilized.

From 30 ml of the pooled fetal serum, 30 mg of α_f-globulin were prepared; from 50 ml of the hepatoma patient serum, 20 mg of α_f-globulin were prepared. The yield of this method determined by radial immunodiffusion test was approximately 70%. The 2 α_f-globulin preparations were homogeneous both by electrophoresis and ultracentrifugation. Fig. 3 shows the pattern of cellulose acetate membrane electrophoresis, and Fig. 4 shows the pattern of disc electrophoresis. By immunoelectrophoresis, both preparations gave a single precipitation line against antiserum to α_f-globulin but did not react with hyperimmune serum to adult human serum (Hyland Laboratories, Los Angeles, Calif.) (Fig. 5). An ultracentrifugal pattern shown in Fig. 6 indicates a high degree of homogeneity.

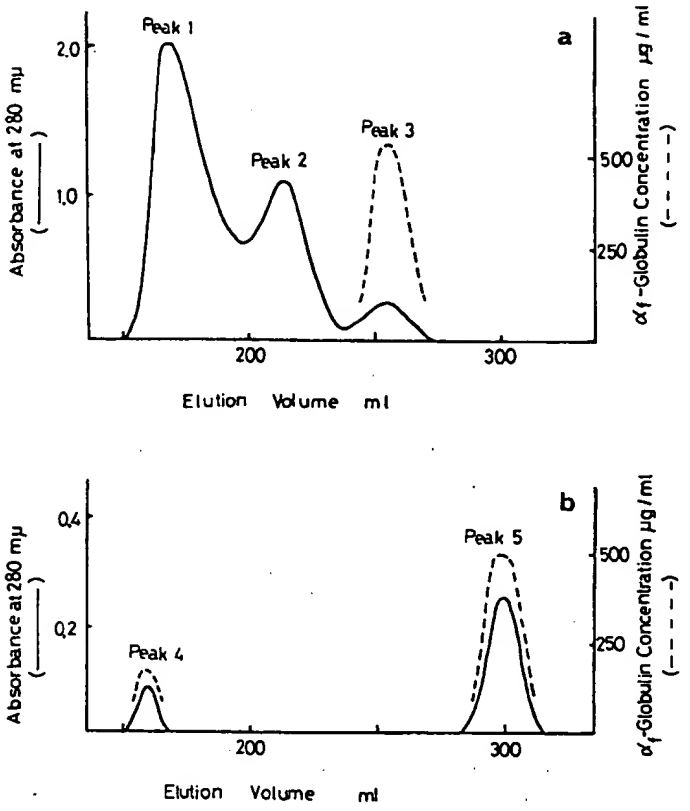

Chart 2. Purification of α_f-globulin from antigen-antibody precipitate prepared from a hepatoma patient serum by gel filtration on Sephadex G-150 column. The 1st gel filtration (*a*) was performed as follows. Eluate, 0.1 M glycine-HCl buffer, pH 1.8; sample, 5 ml of antigen-antibody precipitate (about 150 mg) dissolved in the same buffer; flow rate, 20 ml/hr; column dimensions, 2.6 x 95 cm. The protein concentration was determined by absorbance at 280 mμ. The α_f-globulin concentration was determined by radial immunodiffusion test with the use of antiserum against α_f-globulin. The 2nd gel filtration (*b*) was performed as follows. Eluate, phosphate-buffered 0.15 M NaCl solution, pH 7.0; sample, Peak 3 in the first experiment neutralized and concentrated to 3 ml. Other conditions were similar to the first experiment. Peak 4 consisted of soluble antigen-antibody complex and Peak 5 consisted of antigen. Peak 5 was homogeneous both by electrophoresis and by ultracentrifugation.

Virtually the same result was obtained with the antigen-antibody precipitate from fetuses.

Physicochemical and Chemical Analyses. Analytical data of α_f-globulin preparations both from fetal serum and the hepatoma patient serum were the same, within the errors of the experimental methods.

Some physicochemical data are shown in Table 1. The sedimentation coefficients showed a slight concentration dependency as shown in Chart 3. The partial specific volume which was calculated from amino acid composition and did not include that of carbohydrate portion was 0.726 ml/g (fetus).

α_f-Globulin dissolved in redistilled water gave an ultraviolet absorption spectrum characteristic for a protein with a maximum at 278 mμ. The extinction coefficient $E^{1\%}_{1\,cm}$ (278 mμ) was 5.30 (fetus).

Table 1

Physicochemical properties of α_f-globulin

Source of α_f-globulin	$s^0_{20,w}$ [10^{-13} sec (S)]	$D_{20,w}$ (10^{-7} sq cm/sec)	Molecular weight	$E^{1\%}_{1cm}$ (278 mμ)	$\bar{V}$ (ml/g)
Hepatoma patient	4.50	ND[a]	ND	5.26	0.727
Fetus	4.50	6.18	64,600	5.30	0.726

[a]Not done.

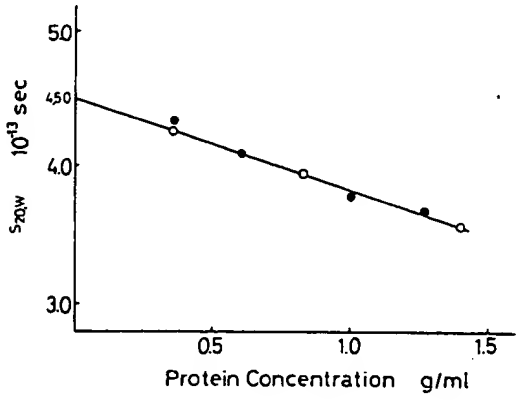

Chart 3. Effect of protein concentration on the sedimentation coefficient of α_f-globulin. The determination was carried out at various concentrations in 0.15 M NaCl solution. The value extrapolated to zero concentrations was 4.50 S (4.50 X 10^{-13} sec). ○, α_f-globulin prepared from a hepatoma patient; ●, α_f-globulin prepared from fetuses.

The contents of nitrogen, sulfur, and nonnitrogenous sugars are shown in Table 2.

The amino acid composition is shown in Table 3. Tryptophan and tyrosine were determined spectrophotometrically as described above. Good agreement was found between the values of tyrosine determined by the automatic amino acid analyzer method and by the spectrophotometric method.

The content of the peptide portion of this protein calculated from the sum of all the amino acid residues was approximately 96%.

DISCUSSION

A marked interest has been taken in this fetal-specific α-globulin in recent years because its reappearance with the primary cancer of the liver not only is of great diagnostic and prognostic value but may offer a new approach to the interpretation of the process of carcinogenesis. However, to date there have been relatively few studies on the physicochemical and chemical structure of this protein, particularly of human α_f-globulin.

Bergstrand and Czar (8) obtained a fraction containing α_f-globulin and albumin by electrophoretic separation of fetal serum. Ultracentrifugal analysis of this fraction showed a single but slightly asymmetric peak. He estimated that this protein has a slightly smaller sedimentation coefficient than that of albumin, which value is 4.6 S.

Gitlin and Boesman (11) estimated that the sedimentation coefficient of this protein is approximately 5.0 S by ultracentrifugation of fetal serum in a sucrose density gradient. He also observed that by gel filtration of fetal serum on Sephadex G-200 this protein was eluted between transferrin and albumin, the molecular weights of which are 69,000 and 90,000, respectively. These observations are in good agreement with the present data.

The presence of fetal-specific α-globulin has been reported in many mammalian species. Among these, only calf fetuin, rat postalbumin, and rat antigen LA were isolated in a high degree of purity and characterized.

Compared to human α_f-globulin, calf fetuin has a smaller sedimentation coefficient (3.47 S), smaller molecular weight (48,400), higher hexose content (8.3%), and quite different amino acid composition (25, 26), and seems to be distinct from human α_f-globulin.

On the other hand, rat postalbumin purified from fetal plasma by Kirsch *et al.* (17) has a sedimentation coefficient of 4.43 S, a diffusion coefficient of 6.25 X 10^{-7} sq cm/sec, and a molecular weight of 64,800. These values are in close agreement to the present data, and postalbumin seems to correspond to human α_f-globulin.

Antigen LA purified from rat amniotic fluid by Stanislawski-Birencwajg (27) has a smaller sedimentation coeffi-

Table 2

Chemical composition of α_f-globulin

Component	Content (1%) Hepatoma[a]	Fetus[b]
Nitrogen	14.9	14.7
Sulfur	1.8	1.7
Nonnitrogenous sugars	3	3

[a]Values determined for α_f-globulin from a hepatoma patient.
[b]Values determined for α_f-globulin from fetuses.

Table 3

Amino acid composition of α_f-globulin

Amino acids were determined on a Hitachi model KLA-3 automatic amino acid analyzer except tryptophan, which was determined spectrophotometrically.

Amino acid	Hepatoma[a]	Fetus[b]	Amino acid	Hepatoma	Fetus
Asparagine	88[c]	87	Methionine	10	8
Threonine	63	65	Isoleucine	46	44
Serine	64	66	Leucine	96	94
Glutamine	186	197	Tyrosine	29	28
Proline	39	38	Phenylalanine	52	48
Glycine	48	47	Lysine	63	64
Alanine	87	90	Histidine	21	22
Half-cystine	24	20	Arginine	30	31
Valine	51	48	Tryptophan	3	3

[a]Values determined for α_f-globulin from a hepatoma patient.
[b]Values determined for α_f-globulin from fetuses.
[c]Values are expressed as moles/1000 moles of amino acids. Good agreement was found between the two values.

cient (2.9 S), a larger extinction coefficient, quite different amino acid composition, and euglobulin nature. In spite of the discrepancy, antigen LA, as well as human α_f-globulin, is one of the most predominant fetal serum α_f-globulins and disappears with an advance of age. In addition, both appear specifically with liver cancer. It is difficult to assess whether or not antigen LA belongs to the same protein species. It would seem, however, possible that antigen LA in serum is identical to postalbumin but somewhat different from that in amniotic fluid.

α_f-Globulin appearing in the serum of hepatoma patients has been reported to be identical to fetal α_f-globulin by an immunological method. α_f-Globulin was prepared from both sera and characterized in the present study. However, no essential difference could be detected between these 2 preparations by physicochemical and chemical criteria.

An immunochemical method was used for purification in this study. This method was simple, and the yield was excellent. Owing to the specificity of antigen-antibody reaction, α_f-globulin was prepared in a high degree of purity from serum which is a complex mixture of various proteins containing only a small amount of this globulin. Purified preparations showed electrophoretic mobility, antigenicity in the double diffusion test, and gel filtration behavior identical to those of α_f-globulin in serum. This protein seems stable at the low pH used in this method.

The amino acid analyses reported in the present study indicate the presence of the usual amino acids found in other serum proteins. The content of the peptide portion, estimated from the recovery of amino acids analyzed and the content of nonnitrogenous sugars, is approximately 97%. Nonnitrogenous sugars are estimated to be present about 3% in the purified material but the possibility of contamination of this specimen with glucose derivatives derived from Sephadex was not completely excluded. Traces of hexosamine could be detected during amino acid analyses. The structure of the carbohydrate components of this protein is under investigation.

ACKNOWLEDGMENTS

I extend my appreciation to Professor Hidematsu Hirai for his excellent instruction and for his council. I also thank Dr. Howard B. Hamilton, Atomic Bomb Casualty Commission, Hiroshima, Japan, for reading this manuscript and Dr. Kunihiko Kobayashi, my colleague, for his valuable discussion.

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Fig. 1. Immunoelectrophoretic pattern of fetal (*F*) and normal adult (*A*) serum against unabsorbed (*aF*) and absorbed (*ab. aF*) rabbit antiserum to fetal serum. Anode is to the *right*. Note reaction for α_f-globulin between fetal serum and absorbed antiserum which is absent between normal adult serum and absorbed antiserum.

Fig. 2. Double immunodiffusion pattern of fetal serum (*F*), normal adult serum (*A*), hepatoma patient serum (H), and α_f-globulin prepared from a hepatoma patient (Ha_f) and from fetuses (Fa_f) against horse antiserum (aa_f) to α_f-globulin prepared by immunization with antigen-antibody precipitate formed between the absorbed rabbit antiserum and a hepatoma patient serum. Note the fused precipitation lines indicating complete antigenic identity and absence of reaction between normal adult serum and the antiserum.

Fig. 3. Cellulose acetate membrane electrophoretic pattern of hepatoma patient serum (*above*) and α_f-globulin from a hepatoma patient (*below*). α_f-Globulin migrated as a single band between albumin and α_1-globulin. Buffer, 0.05 M barbital, pH 8.6; potential gradient, 20 V/cm; time, 45 min. Anode is to the *right*.

Fig. 4. Disc electrophoretic pattern of α_f-globulin from a hepatoma patient. α_f-Globulin was homogeneous, migrating as a single band. Anode is to the *right*.

Fig. 5. Immunoelectrophoretic pattern of α_f-globulin from a hepatoma patient. (Ha_f) and hepatoma patient serum (*H*) against horse antisera to α_f-globulin (aa_f) and to normal human serum (*aA*). Note that no reaction was observed between α_f-globulin and antisera to normal human serum. Anode is to the *right*.

Fig. 6. Ultracentrifugal pattern of α_f-globulin from a hepatoma patient serum. α_f-Globulin was dissolved at 0.8% in 0.15 M NaCl solution. Ultracentrifugation was carried out at 56,100 rpm for 48 min. A single symmetric peak was observed.

Essentially the same results were obtained if α_f-globulin from fetuses were analyzed.

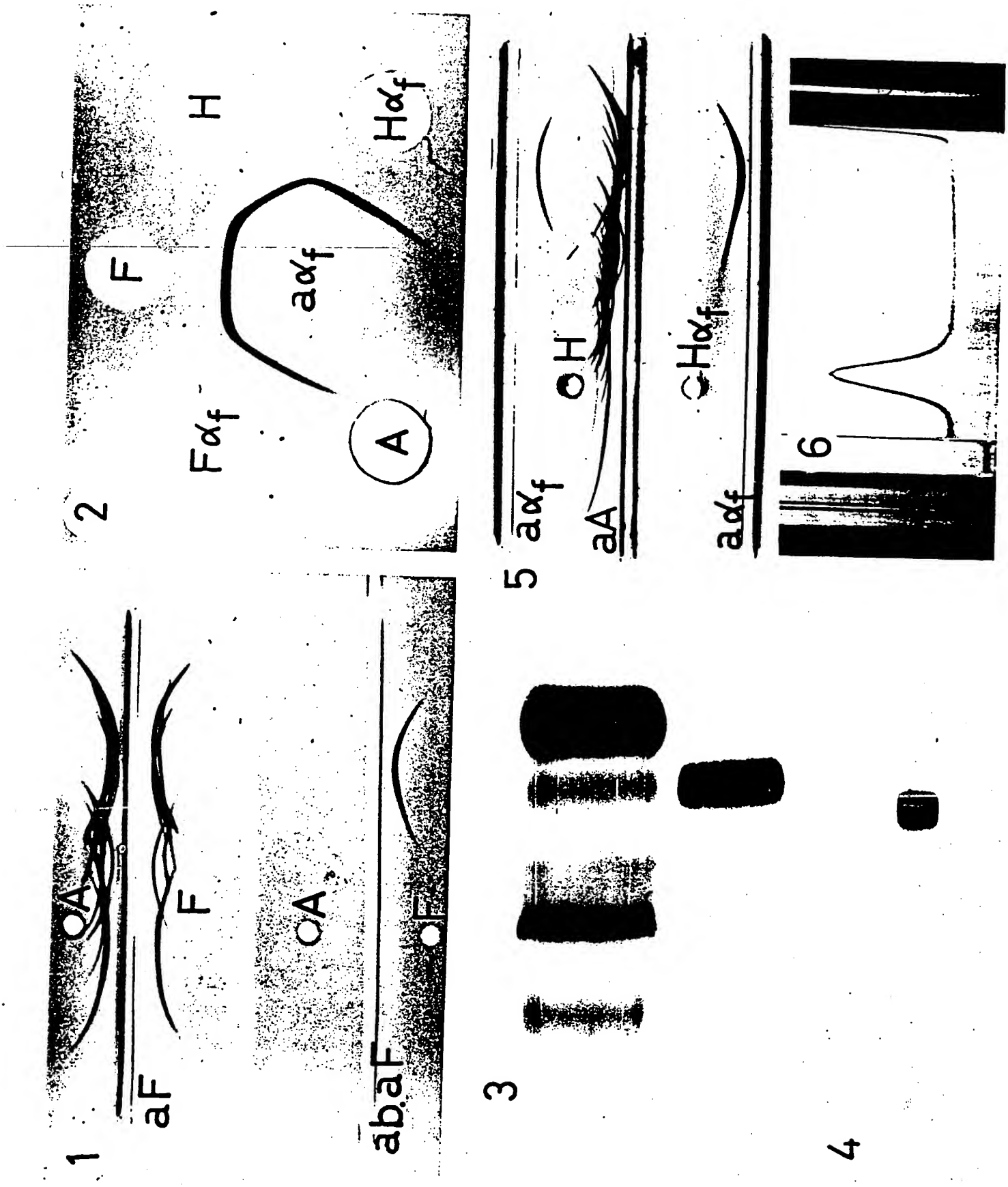
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Table des matières - Inhaltsverzeichnis - Sommario - Contents - Содержание - Indice

Generalia

The carbon dioxide problem. An interdisciplinary survey
1. Introduction
W. Bach 767
2. The carbon cycle: sources and sinks of atmospheric CO_2
G.H. Kohlmaier, U. Fischbach, G. Kratz, H. Bröhl and *W. Schunck* 769
3. The effect of the atmosphere-biosphere exchange on the global carbon cycle
U. Hampicke 776
4. Past and future emission of CO_2
R.M. Rotty 781
5. Prediction of future CO_2 concentrations in the atmosphere
U. Siegenthaler and *H. Oeschger* 783
6. Physiological aspects of increased CO_2 concentration
H. van Keulen, H.H. van Laar, W. Louwerse and *J. Goudriaan* . 786
7. The impact of different energy options on atmospheric CO_2 levels
F. Niehaus 792
8. Climatic effects of increasing atmospheric CO_2 levels
W. Bach 796
9. Measures of CO_2 control
C. Marchetti 806
10. Conclusions
H. Oeschger 808

Specialia

Mathematica, Physica

The critical rotation of gravitating degenerate cylinders
Z.F. Seidov 813

Chimica, Biochimica

Furodendin, a C_{22} degraded terpene from the sponge *Phyllospongia dendyi*
R. Kazlauskas, P.T. Murphy and *R.J. Wells* 814
Synthesis of koenigicine
R.B. Sharma, R. Seth-Verma and *R.S. Kapil* 815
Two 2-dimensional electrophoreses: A combination of crossed immunoelectrophoresis and dodecyl sulfate polyacrylamide gel electrophoresis for protein analysis
T. Yamamoto and *Y. Tanada* 816
Monoamine oxidase inhibitors and histamine metabolism
M. Strolin Benedetti, J.F. Ancher and *N. Sontag* 818
The effect of pH and methylation on the interaction of deoxycholate with rat liver alcohol dehydrogenase
M. Simonetta and *G.M. Hanozet* 820
Co-polymerization of dopa and cysteinyldopa in melanogenesis in vitro
S. Ito, E. Novellino, F. Chioccara, G. Misuraca and *G. Prota* ... 822
The response of cyclic 3',5'-AMP and cyclic 3',5'-GMP phosphodiesterases to experimental diabetes
B. Perez de Gracia, A.R. Rhoads and *W.L. West* 824
A comparison of 3 reagents in converting thiamine to thiochrome in the presence of plant extracts and polyphenols
K. Ratanaubolchai, S. Pikulkarntalert and *B. Panijpan* 825

Biologica

Karyotypes of several frogs from Korea, Taiwan and the Philippines
M. Kuramoto 826
Effect of ethanol and isopropanol on the activity of alcohol dehydrogenase, viability and life-span in *Drosophila melanogaster* and *Drosophila funebris*
Ll. Vilageliu Arqués and *R. Gonzalez Duarte* 828
Spectral sensitivity in a fresh-water Gastrotrich (*Lepidodermella squamatum* Dujardin)
M. Balsamo 830
Identification of intracellular particles of pesticides in ciliate protozoa by Raman microprobe
D. Dive, J.M. Devynck, G. Leroy, M.N. Fourmaux and *Y. Moschetto* 832
Neurosecretory system and storage of paraldehyde fuchsin positive neurosecretory material in the dorsal aorta in an earwig, *Anisolabis annulipes* Lucas (Dermaptera: Labiduridae)
Y.N. Singh and *R. Narain* 834
The effect of RNA synthesis inhibitors on prenucleolar bodies
Gloria Morcillo and *C. de la Torre* 836
Satellite DNA sequences and reproductive isolation in the *Drosophila willistoni* group
G. Corneo, L. Ceccherini Nelli, Donatella Meazza and *F.J. Ayala* 837
Absorption of glycine and proline from the small intestine of rats infected with *Eimeria nieschulzi*
S.J. Ball, Christine E. Heading and *B. Tranter* 839

Physiologica

Effects of altered thyroid state on the inhibition produced by locus coeruleus in Purkinje cells in the rat
Dolores Fuenmayor and *J.A. Gonzalez-Vegas* 841
Microsphere measurement of myocardial capillary bed in hypoxic rats
A.J. Stere, N.W. Brister and *A. Anthony* 842
Catalase activity in electrically stimulated muscle
R.R. Jenkins and *D. Newsham* 843
Effects of inhibition and stimulation of Na^+-K^+ active transport on the resting membrane input conductance of the guinea-pig ventricle
T.M. Nosek 844
Dietary utilization of aliphatic alcohols by *Drosophila*
J. Van Herrewege, J.R. David and *R. Grantham* 846

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the damage of the periventricular structures. An additional advantage of this method is the simplicity and unexpensiveness of the technical tools as compared to the methods using pressure transducers and recording devices[8,9].

1 This work was done under a contract with the Association of Finnish Life Insurance Companies.

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Immunogenicity of agarose-immobilized immune complexes[1]

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Laboratory of Cell Biochemistry, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York (N.Y. 10021, USA), 8 October 1979

Summary. A novel method is described for production of heterologous antisera to a specific tumor-associated murine antigen by immunization with agarose-trapped immune complexes.

Preparation of high titer heterologous antisera to specific tumor-associated antigens is complicated by the use of crude tissue extracts as immunogen in which the relative concentration of the antigen is quite low. Where the tumor-associated antigen is present in moderate quantities, reasonable quality antisera can be obtained by immunization with unfractionated tissue extracts and appropriate serum absorptions. Such antisera have been used to precipitate antigen from solution and the immune complexes, in turn, employed as immunogens to elicit the formation of a more highly specific antiserum[2]. The limiting factors, however, of sufficiently absorbed precipitating antibody, low concentrations of specific antigen in crude tissue extracts and difficulties in the efficient processing of minute precipitates combine to make effective immunization with solution-precipitated immune complexes a prodigious task.

A simple method for the preparation of high titer antisera to a specific tumor-associated antigen, murine γ-FA[3-5], is described. This procedure should be applicable to studies of diverse tissue antigens.

Methods and results. Immunization of rabbits with saline extracts of a mouse fibrosarcoma, antiserum absorption and subsequent identification of an antigen common to tumor, fetal and adult splenic tissue, termed γ-FA, has been described[3-5]. Radial immunodiffusion plates[6] contained 0.1 ml of absorbed anti-γ-FA serum and 2.5 ml of 1% agarose (w/v) in Beckman B-2 buffer, pH 8.6. Antigen wells (3.7 mm in diameter) were cut into the agarose gel, filled with 7 µl of the 10,000 × g supernatant fraction of a 10^{-3} M Tris, pH 7.5, homogenate of normal adult mouse spleen and the plates incubated at 37 °C for 72 h to allow for precipitin ring formation. The agarose slabs were then dialyzed with stirring against daily 200-ml changes of phosphate-buffered saline (PBS) for 3 weeks at 4 °C in order to remove unbound protein. The use of a 1% agarose gel facilitated this removal while antigen-antibody complexes remained trapped within the gel matrix. After dialysis, sections of gel containing precipitin rings were cut out, passed several times through an 18-gauge needle and frozen at −20 °C in twice the volume of PBS. On day 1, 14 and 21, 1 ml of agarose-immobilized immune complexes was emulsified in 1 ml of complete Freund's adjuvant and inoculated s.c. into a New Zealand white rabbit (on day 21 incomplete adjuvant was substituted for complete adjuvant). 22 days later the rabbit was bled and 1-ml aliquots of the antiserum inoculated i.p. into each of several adult C57 mice for in vivo absorption. After 24 h, the mice were bled and the antibody activity of the absorbed antiserum compared with that of the original anti-γ-FA serum. Clearly, the antiserum to γ-FA-anti-γ-FA immune complexes possessed all the precipitin specificity of the original anti-γ-FA serum but at a much higher titer (table) and, unlike the original antiserum, yielded a positive indirect immunofluorescence test[4] on methanol-fixed rat hepatoma cells (figure). This antigen was previously thought to be synthesized only by in vivo propagated tumor cells[4] and the present data, therefore, provide the 1st direct evidence for production of γ-FA by transformed cells. Moreover, reten-

Fig. 1. Indirect immunofluorescence test of in vitro propagated rat tumor cells isolated from a transplanted hepatoma[7]. *A* Original anti-γ-FA serum (1:20); *B* anti-γ-FA immune complexes (1:60). UV light microscopy, BG12-53/44 filters, Zeiss Photoscope.

Table 1. Comparative precipitin tests[a]

Test antigen	Original anti-γ-FA (undiluted)	Anti-γ-FA immune complexes (1:30)[b]
Meth A serum[c]	+	+ + +
Meth A tumor[d]	+	+ + + +
Normal serum[e]	–	–
Adult spleen[f]	+	+ + + +
Viscera[g]	–	–

[a] Hyland Immuno-Plates, pattern 'D'. Preliminary experiments indicated the precipitin line which formed upon interaction of the original anti-γ-FA serum with a saline extract of adult mouse spleen and that which formed by interaction of anti-γ-FA immune complexes with the same splenic tissue extract was one of identity. [b] Calculated dilution after in vivo absorption. [c,d] Serum and saline extract of tumor tissue obtained from a mouse bearing a transplanted 3-methylcholanthrene-induced fibrosarcoma. [e,f] Obtained from normal adult mice. [g] Individual saline extracts of adult mouse liver, kidney, brain, heart, lung, testes and small intestine pooled from several mice.

tion of antibody activity after in vivo absorption in mice (which have γ-FA-positive spleens) suggests that γ-FA is not a cell surface antigen.

Preparation of a high titer anti-γ-FA serum by immunization with agarose-trapped immune complexes will facilitate future in vitro studies of various aspects of cellular transformation. In addition, the method described should be adaptable to diverse tissue antigen systems.

1 Supported in part by a Biomedical Research Support Grant and Grant ROI-CA-20019-04 from the National Cancer Institute.
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The Use of Conventional Antisera in the Production of Specific Monoclonal Antibodies

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(Received 5 May 1983, accepted 27 May 1983)

Monoclonal antibodies specific for human α-2-macroglobulin (α_2M), a plasma glycoprotein, have been produced using a novel immunization method. Commercially available antisera to human α_2M was used to precipitate the antigen from whole serum. Immunization of animals with this immunoprecipitate resulted in the production of hybridomas with a specificity for human α-2-macroglobulin as confirmed by immunoprecipitation.

Key words: *antisera – monoclonal antibody – α-2-macroglobulin – immunization with immune complexes*

Introduction

During the past few years, monoclonal antibodies have become a tool for the detection of cell surface molecules and for the fine structure analysis of macromolecular antigens. As in the initial report of monoclonal antibody production (Kohler and Milstein, 1975), antigen preparations have often consisted of a complex mixture of molecules. Although monoclonal antibodies have the advantage of usually reacting with only one of the components of a heterogeneous mixture, it is often difficult to determine which of the many antibodies produced is actually against the molecule of interest. There is, therefore, a distinct advantage in cases where the immunogen and the antigens used for screening are as pure a preparation as possible. In cases where a conventional antisera against the antigen of interest is available, immunization with the antigen precipitated with this serum can be an effective way to produce monoclonal antibodies.

We have chosen to use this method to produce specific monoclonal antibodies against human α-2-macroglobulin (α_2M). α-2-macroglobulin, a glycoprotein pro-

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tease inhibitor in human plasma, is present in quantities ranging from 1.5 to 2 mg/ml depending upon the age and sex of the individual (Laurell and Jeppsson, 1975). Using goat anti-human α-2-macroglobulin serum, human α_2M was precipitated from serum and injected into animals. Hybridomas produced by the fusion of Sp2/0-Ag14 and sensitized spleen cells were screened for their specificity of reaction by an adaptation of the enzyme-linked immunosorbent assay reported by Engvall and Perlmann (1971).

Materials and Methods

Parental cell line

Sp2/0-Ag14 (Shulman et al., 1978), a non-immunoglobulin producing mouse plasmacytoma cell line, was maintained in Dulbecco's Modified Eagle's Medium with high glucose (DMEM-Hg) supplemented with 10% calf serum and glutamine.

Preparation of protein for immunizations

α-2-macroglobulin (α_2M) was isolated from human serum using anti-human α_2M (Cappel Laboratories, Cochranville, PA). Both serum and antiserum were stored at −70°C. The antibody-antigen complex obtained from the mixture of the antiserum and serum was prepared in double diffusion plates (Kallestad, Chaska, MN). After extensive washing in phosphate buffered saline (PBS), pH 7.2, to remove other proteins from the gel, the precipitin bands were excised from the surrounding gel.

Immunization procedure

The precipitin bands were homogenized, resuspended in PBS and injected into female BALB/c mice (Ace Animal Suppliers). Material from six bands (i.e. the amount of α_2M precipitated from 15 μl of serum) were injected into mice at weekly intervals for 3–8 weeks. The route of immunization alternated between intraperitoneal and subcutaneous injections. For the 3 days prior to the fusion, the mice were given daily intravenous injections of the immunoprecipitate containing twice the amount of protein given on previous occasions. The same procedure was followed for immunization of rats although the amount of antigen in each immunization was doubled. On the day of the fusion, the spleen was removed aseptically and blood was collected for titration of anti-α_2-macroglobulin antibodies. This serum was used as a control serum in ELISA assays.

Fusion

The fusion, cloning, and maintenance of the hybrid cells were performed according to the method reported by Kennett et al. (1978).

ELISA screening assay

Screening for those hybridomas producing immunoglobulin specific for human α_2M was modified from a technique described by Kennett (1980). The source of the human α_2M was an immunoprecipitate resulting from the reaction of human serum

(containing 0.1 mg soybean trypsin inhibitor (Sigma) and 500 KIU (kallikrein inactivator units) of Trasylol (aprotinin) (FBA Pharmaceuticals) per milliliter) with the goat antiserum. The precipitated protein was washed three times in PBS, diluted in 0.05 M sodium carbonate buffer, pH 9.8, and distributed to the wells of a 96-well protein binding polystyrene plate (Microelisa plates, Dynatech Laboratories, Inc., Alexandria, VA). Control plates were prepared using goat anti-human α_2M alone. After the plates had been incubated at 4°C for 18 h, they were washed twice by immersion and flicking with a 0.05% Tween 80 (Sigma) solution in PBS. Monoclonal antibody was added and incubated for 2 h at room temperature. Following several washings in Tween 80/PBS, the peroxidase conjugated goat anti-mouse immunoglobulin (Cappel Laboratories), absorbed with an equal volume of purified human immunoglobulin (Cappel Laboratories) to remove cross-reacting antibodies and diluted in Tween 80/PBS containing 0.05 M EDTA and 2% additional NaCl, was added to the wells. The plates were incubated for two additional hours at room temperature and washed 5–7 times in Tween 80/PBS. After the final wash, 150 μl of solution of 5.5 mM *o*-phenylene diamine, 0.012 M hydrogen peroxide, dissolved in 0.1 M citrate buffer, pH 4.5, was added to each well. The enzyme reaction liberating a colored product was allowed to proceed for 20–30 min before stopping the reaction with 0.1 M sodium fluoride. The color was read quantitatively on a Multiscan spectrophotometer (Flow Laboratories) at 450 nm.

Determination of immunoglobulin class

Class of immunoglobulin was determined by precipitation using class specific antisera (Miles Laboratories, Elkhart, IN) in double diffusion gels.

Ammonium sulfate precipitation of immunoglobulin

Hybridomas were grown in mass culture in DMEM-Hg supplemented with 5% calf serum. Cells were grown to mid-log phase and maintained until 50% of the cells were unhealthy and supernatant harvested. Equal volumes of saturated ammonium sulfate solution and culture supernatant were mixed. The precipitate was spun at 48,000 × *g*, dialyzed against PBS, and frozen at −70°C (Jonak, 1980).

Competition assay

Actively growing cultures of hybridomas were labeled with L-[^{35}S]methionine (Amersham U.K.) after being washed in methionine-deficient RPMI 1640 (Grand Island Biological Co., NY) supplemented with 30 mg/ml glutamine (Sigma) and 5% fetal calf serum. A volume of 250 μl of [^{35}S]methionine was added to 10^7 cells and incubated for 18 h in a 5% CO_2 incubator. Twenty minutes before harvesting the supernatant, 0.1 ml of 10 mM methionine was added.

After overnight incubation at 4°C, 2.5 μg of α_2M immunoprecipitate dissolved in 0.2 M phosphate buffer, pH 8.0, was attached per well of 96-well polyvinylchloride (PVC) plates (Dynatech). The PVC plates were blocked for 1 h with incubation buffer containing 0.1 M phosphate buffer, 0.15 M NaCl, 0.02% NaN_3, 0.25% BSA and 2.0% calf serum. Unlabeled antibody in the form of dense culture supernatant was added (50 μl/well) and incubated for 2 h. Then 50 μl of biosynthetically labeled

[^{35}S]methionine monoclonal antibody was added and incubated for an additional 2 h. The wells were washed, dried and counted.

Monoclonal antibody immunoprecipitation

Formalin-fixed *Staphylococcus aureus* (Calbiochem) coated with goat anti-mouse immunoglobulin (Cappel) was washed and incubated with monoclonal antibodies according to the method of Lampson (1980). After the incubation with antibody, antigen in the form of whole human serum containing soybean trypsin inhibitor and Trasylol in concentrations mentioned above was incubated for 18 h with *S. aureus*-antibody complex. After washing, the antibody-antigen complex was eluted from the *S. aureus* by boiling for 2 min in sample buffer containing 0.125 M Tris (Sigma), 2.2 M glycerol (Mallinckrodt), 0.2% Coomassie Brilliant Blue R250 (BioRad), 0.16 M sodium dodecyl sulfate (SDS)(BioRad), and 10% β-mercaptoethanol (Sigma). The samples were spun in a Beckman ultrafuge and applied to a 5% acrylamide-0.13% bis-acrylamide gel with 0.1% SDS and run at constant current. The gel was subsequently stained with 0.2% Coomassie Brilliant Blue R250 in methanol-water-glacial acetic acid (5 : 5 : 1), destained and dried on a gel slab dryer (BioRad).

Starch gel electrophoresis of treated human serum

Samples of goat anti-human α_2M and *S. aureus* coated with anti-mouse immunoglobulin and monoclonal antibodies were incubated with human serum plus inhibitors for 18 h at 4°C. The solutions were spun down and applied to Whatman No. 3 filter paper strips. Horizontal starch gel electrophoresis (Poulik system) was performed as described by Harris and Hopkinson (1976). The gel containing 13% starch (Electrostarch, lot 307) was prepared in gel buffer (76 mM Tris/citrate, pH 8.6) and the bridge buffer, 0.3 M boric acid, brought to pH 8.0 with NaOH. Sample-saturated filter paper strips were inserted into the gel. Electrophoresis was carried out for 6 h at 7 V/cm at room temperature. The gels were stained for protein with 0.02% nigrosin (Sigma).

Results

Hybridomas were produced in four fusions (Table I) using animals that were immunized with a goat immunoglobulin-human α_2-M complex. Animals were immunized by an adaptation of the procedure reported by Stahli et al. (1980). Supernatants from these hybridomas were assayed quantitatively by the ELISA method for antibody binding to protein attached to polystyrene plates. Monoclonal antibody activity was detected by peroxidase-conjugated goat anti-mouse immunoglobulin and the appropriate substrate. As shown in Table I, all fusions produced antibodies that reacted with goat immunoglobulin. However, only fusions 1, 3 and 4 produced antibodies with a presumed specificity for human α_2M. Fig. 1 illustrates the degree of binding of individual antibodies to equal amounts of both the α_2M immunoprecipitate and the antisera alone. These antibodies demonstrate a significantly greater degree of binding to the α_2M precipitate than the precipitating

TABLE I

PRODUCTION OF ANTIBODIES AGAINST HUMAN α_2M-GOAT IMMUNOGLOBULIN COMPLEX

Fusion	Origin of spleen cells	Immunization protocol	Total	Number of hybrids		Hybrids with activity to α_2M after 30 days in culture (%)
				Anti-goat Ig (%)	Anti-α_2M (%)	
1	BALB/c	3 s.c./i.p. 1 i.v.	34	4 (12%)	6 (18%)	0
2	BALB/c	8 s.c./i.p. 3 i.v.	44	2 (4.5%)	0	–
3	BALB/c	8 s.c./i.p. 3 i.v.	313	16 (5%)	39 (12.5%)	18 (46%)
4	Brown Norway	6 s.c./i.p. 3 i.v.	316	41 (13%)	30 (9.5%)	8 (27%)

Abbreviations: i.p. = intraperitoneal; i.v. = intravenous; s.c. = subcutaneous.

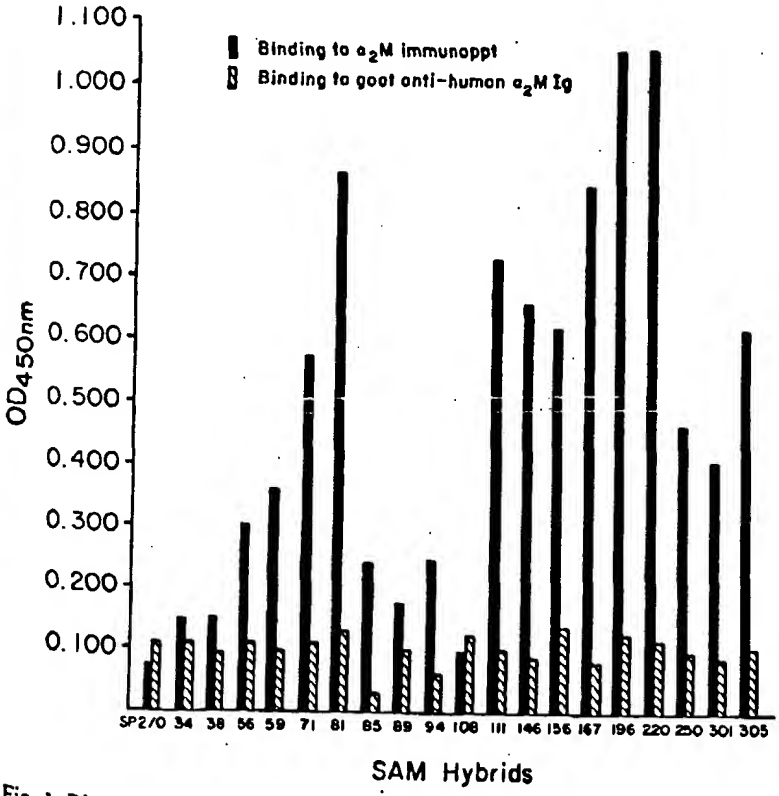

Fig. 1. Binding of mouse anti-α_2-macroglobulin (SAM hybrids) supernatants to immunizing proteins. Bar graphs are representative of duplicate samples from a single assay. Immunoprecipitate and immunoglobulin were bound to wells of a polystyrene plate and tested for the binding of supernatants to the 2 proteins by the ELISA method. Shown here are those monoclonal antibodies that react with the immunoprecipitate but do not bind significantly to goat immunoglobulin.

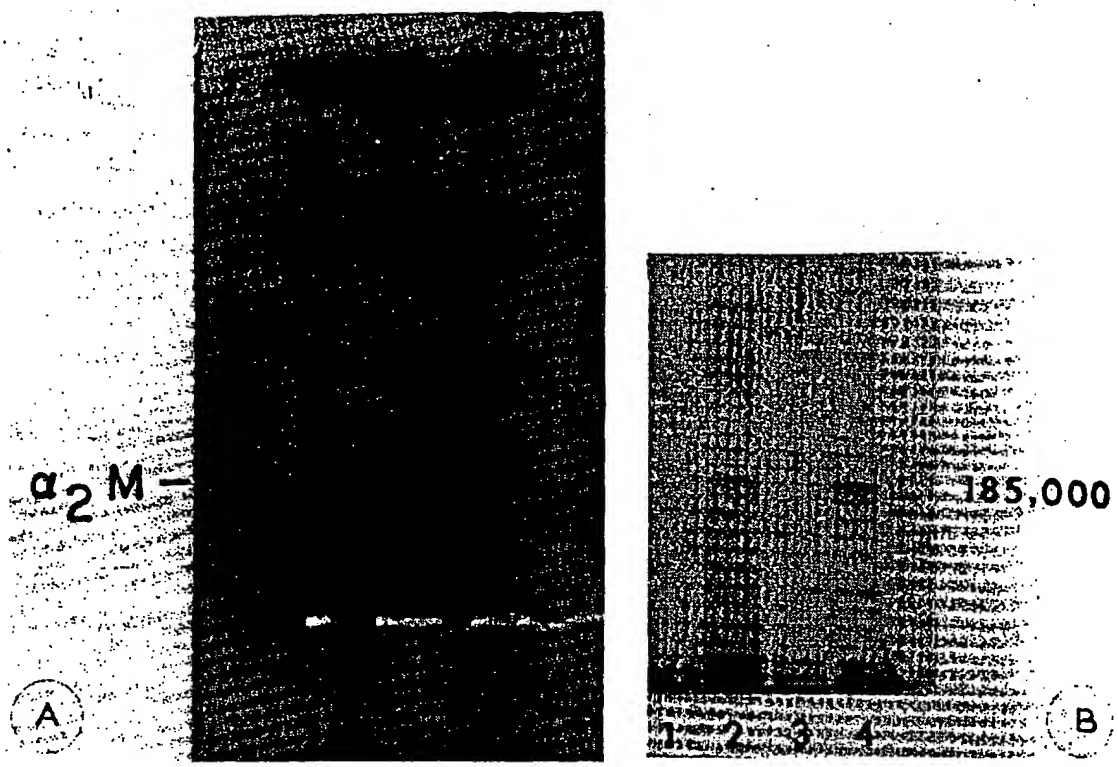

Fig. 2. Confirmation of specificity of anti-α_2-macroglobulin monoclonal antibodies. A: Removal of α_2M from human serum separated by starch gel electrophoresis. Formalin-fixed *S. aureus* was coated with anti-mouse immunoglobulin, pre-incubated with Sp2/0-Ag14 cell culture supernatant or a pooled sample of 16 anti-α_2M monoclonal antibodies concentrated by ammonium sulfate precipitation and incubated with diluted human serum. Samples were centrifuged and the supernatant applied to a 13% starch gel (Poulik system) using filter paper wicks, electrophoresed for 6 h and stained with nigrosin. Lane 1 is diluted human serum. Lanes 2 and 3 show the proteins remaining in human serum after treatment with pre-coated *S. aureus* that had been incubated with immunoglobulin. Lane 2 is human serum treated with Sp2/0-Ag14 supernatant. Lane 3 is human serum treated with pooled monoclonal antibodies. Note the removal of α_2M from serum that has been treated with the anti-α_2M monoclonal antibodies. B: Immunoprecipitation of α_2-macroglobulin from human serum. Formalin-fixed *S. aureus* was coated with goat anti-mouse immunoglobulin and incubated with parental plasmacytoma supernatant or monoclonal antibodies as described above. The antibody-antigen complex was removed from the *S. aureus* by boiling for 2 min with a reducing agent added. *S. aureus* was spun and the supernatant applied to a 5% bis-acrylamide SDS gel. Lane 1 contains the molecular weight standard. The remaining lanes contain the immunoprecipitates found using: pooled supernatants from 16 hybridomas making monoclonal anti-α_2 macroglobulin (lanes 2 and 4) and Sp2/0-Ag14 cell culture supernatant (parental plasmacytoma) (lane 3).

antisera. More than 50% of the hybridomas with a detectable reactivity to human α_2M lost this activity as the cultures were expanded during the 4 weeks following the initial assay (Table I). Hybridomas which did not lose antibody activity during that time were cloned in semi-solid agarose.

The reaction of 16 mouse monoclonal antibodies with α_2M was confirmed by the immunoprecipitation of the protein. Antibody was pooled from 16 mouse hybridomas and concentrated by ammonium sulfate precipitation before reaction with anti-mouse immunoglobulin-coated *S. aureus*. This coated *S. aureus* was then incubated with human serum to remove the corresponding antigen. After centrifuga-

tion of the *S. aureus*-antibody complex, the supernatant was applied to a 13% starch gel and electrophoresed. Comparison of human serum before and after treatment with the pooled antibodies shows the removal of the protein band corresponding to the migration pattern of α_2M in starch gel electrophoresis of human serum treated with the monoclonal antibody-*S. aureus* complex (lane 3, Fig. 2A). Alternatively, confirmation of this specificity by SDS-polyacrylamide gel electrophoresis of substances precipitated from human serum treated with monoclonal antibody-*S. aureus* complex demonstrate the precipitation of a single protein with a molecular weight of approximately 185,000 (lanes 2, 4, Fig. 2B). This is the molecular weight of the reduced subunit chain of α_2M (Harpel, 1973). Further confirmation of the specificity of the monoclonal antibodies was obtained by reaction of the individual monoclonal antibodies which comprised the pooled antibody mixture with purified α_2M. α_2M purified from human plasma according to the procedure of Kaplan et al. (1981) was used as the antigen in the ELISA procedure. All antibodies demonstrated significant binding to the purified protein (data not shown).

The antibody class of 18 independent mouse antibodies was determined by immunoprecipitation with class specific antisera. Three hybridomas produce IgM antibodies, 13 produce IgG_1 antibodies, one produces IgM and IgG_1, and one produces IgG_1 and IgG_{2a}. The two hybridomas which produce two classes of antibody were uncloned hybrids. An estimate of the antigenic determinants identified by these antibodies was determined by competitive binding inhibition assays as described in Materials and Methods. These assays indicate that a minimum of 7 sites are identified by 13 monoclonal antibodies (data not shown).

Discussion

A major advantage in using monoclonal antibodies is that one can isolate a single antigen from a complex mixture. A simple purification of the antigen often simplifies the monoclonal antibody screening procedure. We have demonstrated that the use of conventionally produced antisera is advantageous in isolating antigens that will ultimately be used for the immunization of animals for hybridoma production. The goat immunoglobulin-human α_2-macroglobulin immune complex acted as an effective immunogen in both rats and mice. Consequently, we were able to demonstrate the successful production of stable monoclonal antibody producing lines with a specificity to human α_2M.

This method has also been used to produce monoclonal antibodies to human complement components (Jonak, personal communication). Several advantages are apparent in using this technique. First, if a conventional antiserum is already available, tedious purification of the antigen for immunization is eliminated. Second, purification of the antigen for the screening procedure is not necessary since antibodies can distinguish between the immunoprecipitate and the antisera alone. Third, since conventional antisera have been produced against a wide variety of proteins and other biological substances, this technique provides an effective method for the study of these same molecules by monoclonal antibodies.

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